THE FINE STRUCTURE OF THE MATURE SPERM OF THE FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII*

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ABSTRACT

Electroejaculated spermatophores from male *Macrobrachium rosenbergii* contain mature sperm which resemble everted umbrellas. The nonflagellated, nonmotile sperm consist of a 10 μm main body (base) and a single 12–15 μm spike extending from the convex surface of the base. The nucleus is flocculent (decondensed), contains basic proteins, is not delimited by a nuclear membrane, and is housed in the base. The cytoplasm of the convex base contains fifteen to twenty radial fibrils which anastomose to form the spike. Both the spike and the radial fibrils are cross-striated with a periodicity of 35 nm. These structures contain 6 nm filaments running perpendicularly to the cross-striations. A pair of centrioles composed of nine doublet tubules are located acentrically near the origin of the spike in the cytoplasm of the base. Radial fibrils are interconnected to the centriolar matrix by accessory fibrils. No clearly definable acrosomal region is identifiable morphologically or histochemically.

INTRODUCTION

Considered atypical due to their lack of a flagella and midpiece, decapod crustacean sperm attracted early investigators and prompted numerous light microscopic studies (*e.g.*, Labbe, 1903; Koltzoff, 1906; Retzius, 1909; Binford, 1913; Fasten, 1924; Bowen, 1925; Nath, 1937; Worley, 1939; McCroan, 1940). Often these early reports were directed at determining homologies between the nonflagellated crustacean sperm and the flagellated sperm of other animals. Later investigators have continued the morphological studies on the nonmotile decapod sperm using electron microscopy and extensive histochemical techniques (*e.g.*, Ruthman, 1958; Yasuzumi, 1960; Moses, 1961; Pochon-Masson, 1963, 1969; Brown, 1966; Hinsch, 1969; Talbot and Summers, 1978; Koehler, 1979). Morphological descriptions have resulted in the crustacean decapod gametes being divided into two classes, the unistellate sperm of the natantians (shrimps) (Clark *et al.*, 1973; Brown *et al.*, 1976; Lu, 1976; Talbot and Summers, 1978), and the multistellate sperm of the reptantians (lobsters, crabs, crayfish) (Moses, 1961; Brown, 1966; Hinsch, 1973; Talbot and Summers, 1978). These studies have shown the decapod sperm consists basically of a spherical main body with a variable number of radiating appendages (Wilson, 1928; Talbot and Summers, 1978). The main body is generally believed to house the nucleus, acrosome, and a lamellar region, though variations of this scheme have been documented (Yudin *et al.*, 1979).

Although numerous light and fine structural studies are available on the structure of the natantian sperm (Koltzoff, 1906; Retzius, 1909; Nath, 1937; Pochon-Masson, 1968b, 1969; Clark *et al.*, 1973; Koehler, 1979; Lu, 1979; Yudin *et al.*, 1979; Kleve *et al.*, 1980), natantian sperm are still poorly understood as compared to the reptantian sperm. Functions have been demonstrated for many of the structures in the reptantians (Brown, 1966; Hinsch, 1971; Talbot and Chanmanon, 1980; Goudeau, 1982) and in the Penaeidae natantians (Yudin *et al.*, 1979; Clark *et al.*, 1980). Brief reports on the morphology of the mature sperm of the Caridea natantians have illustrated the great diversity of sperm structures between the palaemonids and penaeids (Pochon-Masson, 1969; Lynn, 1981; Sandifer and Lynn, 1981; Sellos and Le Gal, 1981). As an integral part of fertilization studies in the freshwater caridean shrimp *Macrobrachium rosenbergii,* a thorough description of the mature sperm using electron microscopy and histochemical techniques is presented here.

MATERIALS AND METHODS

Male *M. rosenbergii* were obtained from the Institute of Marine Resources, Charleston, South Carolina, and held individually in compartmentalized 45 gallon fiberglass tanks. Aerated flow-through water was maintained at 25–28°C.

Spermatophores containing mature sperm were obtained from males by electroejaculation (Sandifer and Lynn, 1981). Isolated free sperm were obtained by homogenization of spermatophores in a 20% MBL sea water solution (Cavanaugh, 1956) with a pyrex ground glass tissue homogenizer (0.15 mm clearance). A subsequent 5–10 min incubation with 0.01% amylase or 0.01% diatase (Sigma) in a Tris buffer (pH 7.4) eliminated much of the acellular spermatophore contamination. This suspension was layered on a 10% sucrose solution and centrifuged at 2000 × g for 10 min. The resulting pellet was resuspended and washed four times with Tris buffer. Sperm were examined with phase contrast and differential interference phase contrast optics.

Whole sperm were analyzed histochemically using acridine orange, periodic acid-Schiff (PAS) reagent, Feulgen and basic fast green stains. For acridine orange, sperm suspensions were treated live (Thompson, 1966) and examined on an Olympus epiflourescent microscope. Sperm suspensions were also fixed in 0.1 M PO_4 buffered 10% formaldehyde at pH 7.4. These sperm suspensions were stained with PAS and Feulgen reagent (Thompson, 1966), basic fast green (Alfert and Geschwind, 1953), and acid fast green (Langreth, 1969).

For scanning electron microscopy (SEM), spermatophores were compressed between two coverslips and fixed in 4% glutaraldehyde buffered with 0.1 M PO_4 at pH 7.4 for 20 min. After postfixation, samples were rapidly dehydrated in a graded acetone series and critically point dried with CO_2. Samples were mounted on SEM studs and coated with 20 nm gold in a Polaron sputter coater with a rotating stage. Sperm were examined on a Philips SEM 501 microscope at an accelerating voltage of 10 kV.

For transmission electron microscopy (TEM), free sperm suspensions and spermatophores were fixed for two hours in a 0.1 M PO_4 buffered 4% glutaraldehyde solution containing 1% tannic acid at pH 7.4. Sperm were washed in 0.1 M PO_4 buffer, postfixed 30 min in 0.2 M PO_4 buffered 0.5% osmium tetroxide, washed again in PO_4 buffer, rapidly dehydrated in a graded acetone series, and embedded in a low viscosity epoxy resin (Spurr, 1969). Sections were cut with a diamond knife on a Sorvall MT-2B ultramicrotome, stained with saturated methanolic uranyl acetate and aequeous lead citrate (Venable and Coggeshall, 1965), and examined on a Hitachi HU11E electron microscope at an accelerating voltage of 75 kV.

Spermatophores

A single pair of spermatophores may be obtained from a mature male once a day using electroejaculation. As the spermatophores are extruded from the terminal ampullae through the gonopores at the base of the fifth pair of walking legs, the distal tips contact and the spermatophores join and adhere as they move posteriorly. After a brief period of hydration (10–30 min), the spermatophores from *M. rosenbergii* are easily differentiated into two morphologically distinct regions (Fig. 1). In the fused condition, two white opaque strips containing the mature sperm are observed on the lateral edges of the spermatophore complex. Proximal to the fused medial line are two thicker, more translucent regions. These regions contain no sperm and can be mechanically removed to reduce the cellular and acellular contamination they otherwise contribute to sperm suspensions. The remaining spermatophore matrix is PAS positive and is largely removed by treatment with amylase or diatase. Enzyme treatment does not appear detrimental to the sperm.

General sperm morphology

Mature *M. rosenbergii* sperm isolated from the spermatophore mass are non-motile and resemble an "everted umbrella" when observed with phase microscopy (Fig. 2). The main body (base) of the sperm is slightly cupped and a single appendage (spike) projects from the convex surface of the base. The peripheral edges of the sperm base have a ruffled or irregular appearance. Fourteen to twenty radial fibrils converge at the center of the base (Fig. 3). At the point of convergence and immediately beneath the spike is a highly birefringent locus.

In SEM, the radial fibrils can be clearly seen anastomosing into the spike (Fig. 4). The radial fibrils have a diameter of 225 nm and the 12–15 μm long spike tapers from approximately 850 nm at the base to 350 nm at the tip. Slightly irregular in shape, the base of the sperm is 9–10 μm in diameter and approximately 4 μm in height (Fig. 4, 5). The sperm can be divided into three morphologically distinct regions when examined at the fine structural level (Fig. 6). These regions which will be discussed in the following order are the base, the spike, and the cap-like area on the convex surface of the base.

Morphology and cytochemistry of the main body

The base contains the nucleus, identified as a Feulgen-positive region. Sperm cells stained with acridine orange exhibit a green flourescence in the base, further indicating double stranded nucleic acids. Basic proteins are also associated with and confined to the nuclear region as indicated by basic fast green staining. With TEM, the nucleus appears as a flocculent, decondensed area (Fig. 6) and almost entirely fills the cup-shaped base. The nucleus is not limited by a nuclear envelope, but rather by the plasmalemma on one side and the membrane bound region of the cap on the other. In tannic acid-glutaraldehyde fixations, the plasmalemma around the nuclear cup is relatively smooth (Fig. 6).

Spike morphology and cytochemistry

The spike of the sperm is composed primarily of protein as indicated by positive acid fast green staining and digestion of the spike by trypsin. This region of the cell does not exhibit a positive reaction to PAS.
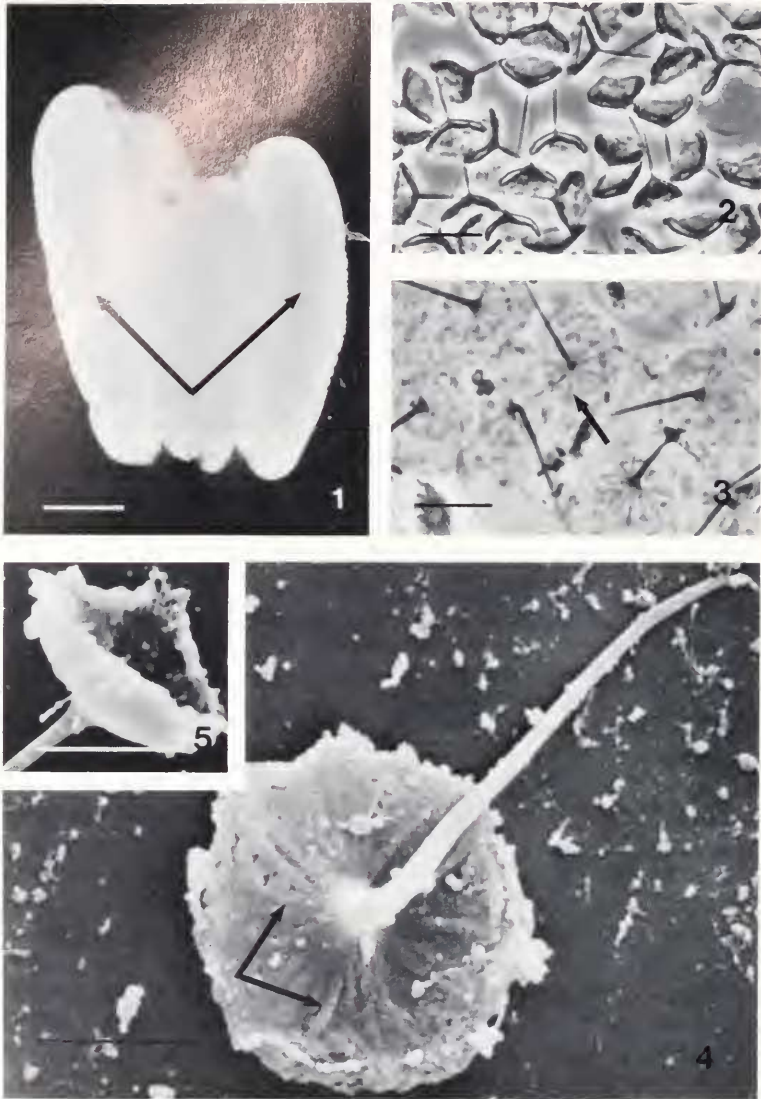
FIGURE 1. Fused electroejaculated spermatophore pair showing medial translucent region and the lateral opaque region (arrows) which normally contain the mature sperm. Bar = 1 mm.

FIGURE 2. A phase micrograph of mature *Macrobrachium rosenbergii* sperm showing the normal everted umbrella configuration. Bar = 10 μm.

FIGURE 3. A phase micrograph of mature sperm showing the radial fibrils (arrow) in the sperm base which anastomose to form the spike. Bar = 10 μm.

FIGURE 4. A scanning electron micrograph more clearly illustrating the anastomosing radial fibrils of the base. Bar = 5 μm.

FIGURE 5. The distinct cup shape of the base with the spike projecting from the convex surface is shown. Bar = 5 μm.

Viewed with TEM, the spike is continuous with the radial fibrils of the cap (Fig. 6). At high magnifications, the spike has a cross striated appearance, the striations running perpendicular to the length of the spike (Fig. 7). The cross striations have
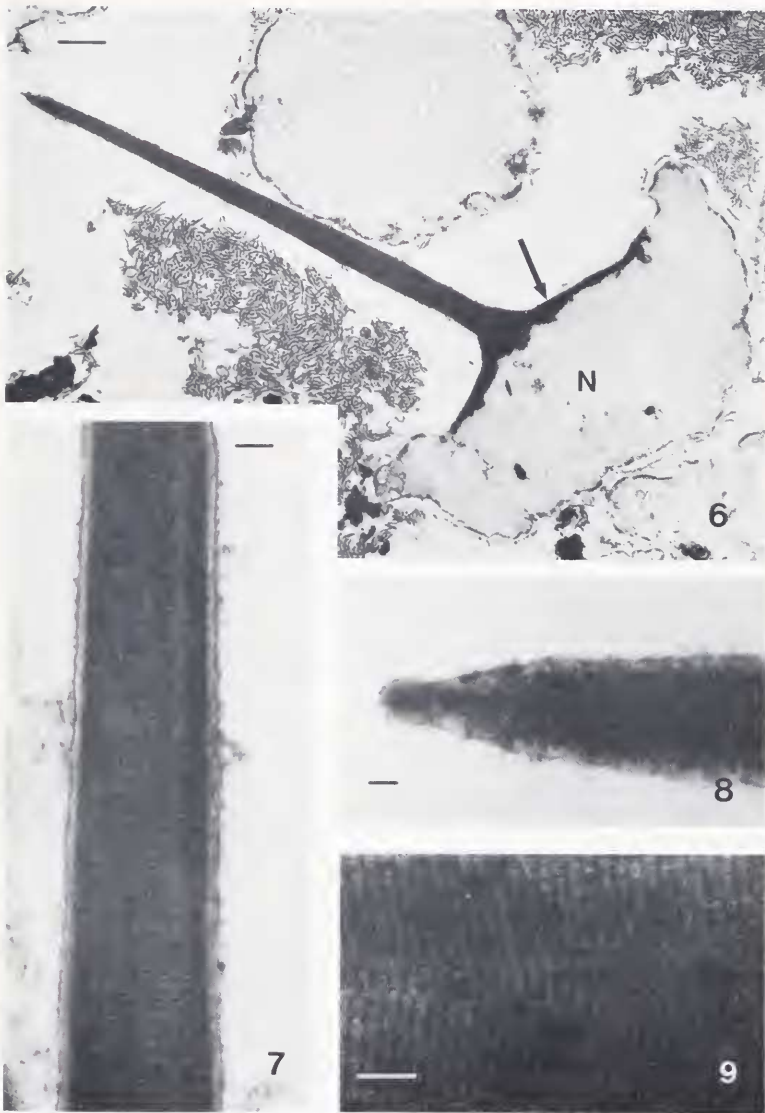
FIGURE 6. At the fine structural level, a longitudinal section through a mature sperm shows the decondensed nuclear region (N) in the base, the caplike region (arrow) housing the radial fibrils, and the cross-striated spike. Bar = 1 μm.

FIGURE 7. The cross striated appearance of the spike is apparent at higher magnifications. Note the numerous 6 nm filaments running perpendicular to the cross-striations. Bar = 0.1 μm.

FIGURE 8. The spike terminates in a 50 nm bulbous tip. Bar = 0.1 μm.

FIGURE 9. Occasionally the cross striations of the spike are observed to be out of register, indicative of the anastomosing radial fibrils of the cap-like region. Bar = 0.1 μm.

a 35 ± 2.5 nm periodicity. Numerous 4–7 nm filaments run longitudinally between the dense bands of the cross striations and both filaments and cross striations run the length of the spike terminating in a 50 nm spike tip (Fig. 8). The cross striations of the spike are occasionally out of register (Fig. 9), indicative of the anastomosing radial fibrils from the cap.

Oblique sections close to the base illustrate the anastomosing fibrils (Fig. 10). Although striations are often apparent in the oblique sections, the periodicity may appear compressed due to the angle of section. Sections through the spike midway between the tip and the base are often amorphous and structural elements are difficult to resolve. Closer to the tip, however, tubule-like structures with an outside diameter of 26 nm are observed (Fig. 11). Such microtubular structures are observed only in cross sections at or near the tip of the spike.

Cap region morphology and cytochemistry

Fifteen to twenty radial fibrils that anastomose to form the sperm spike comprise the major substructure of the cap (Figs. 6, 12). The cap region demonstrates a weak, nonspecific reaction to PAS. The fibrils react positively with acid fast green indicating a proteinaceous nature. When viewed at the fine structural level, the radial fibrils are continuous with the spike and have the identical, periodic cross striation and 4–7 nm filaments (Fig. 12).

The cap region, including a thin band of cytoplasm, appears to be at least partially separated from the nucleus by a membrane which appears double in some areas (Fig. 13). Whether the membrane system limiting the cap is complete and fuses with the single plasmalemma surrounding the sperm is presently unclear. In the cytoplasmic band of the cap, a pair of centrioles are housed acentrically in relation to the base of the spike (Figs. 13, 14). The centriolar pair is embedded in a dense matrix. Dense bodies are observed in association with the centrioles and are apparently sections through the cross striated fibrils associated with the centriolar matrix (Fig. 16). These fibrils intersect this matrix independent of the centrioles' orientation (Figs. 13, 14) and extend between the centriolar matrix and the radial fibrils of the cap. Each centriole is approximately 160 nm in diameter and 225 nm in length consist of nine doublet microtubules (Fig. 15) with a pair of dynein-like arms attached to each doublet. A flocculent material occupies the central core and appears to have a spoke-like arrangement attached to each of the doublets.

DISCUSSION

Although some fine structural differences are present, the sperm of *M. rosenbergii* conform to the basic morphology reported in other caridean shrimp (Nath, 1937; Pochon-Masson, 1968b, 1969; Koehler, 1979; Sellos and Le Gal, 1981). The mature sperm are nonmotile and comprise a cup-shaped base housing the nucleus and a single spike which projects from the convex surface of the base.

The nucleus is decondensed, as is typical of the decapod crustacean sperm, and a nuclear envelope is absent as in other natantians (Pochon-Masson, 1969; Clark *et al.*, 1973; Lu, 1976; Talbot and Summers, 1978; Kleve *et al.*, 1980; Goudeau, 1982). Basic proteins are associated with the nucleus in *Macrobrachium* sperm. Basic proteins have also been reported in other decapod sperm (Bloch, 1966; Vaughn, 1968; Koehler, 1979; Sellos and Le Gal, 1981) but may be absent in some cases (Chevaillier, 1966; Lu, 1976; Kleve *et al.*, 1980). The basic proteins observed in the sperm of the *Palaemon serratus* have been identified as histones with no protamines present (Sellos and Le Gal, 1981). The significance of the basic proteins in some decapod sperm but not in others is presently unclear.

The nucleus extends to the plasmalemma of the base with no intervening region of cytoplasm as shown in *P. serratus* (Sellos and Le Gal, 1981). It should be noted, however, that membranous blebbing and whorls are found associated with the plasmalemma when *M. rosenbergii* sperm are fixed with paraformaldehyde or glutar-
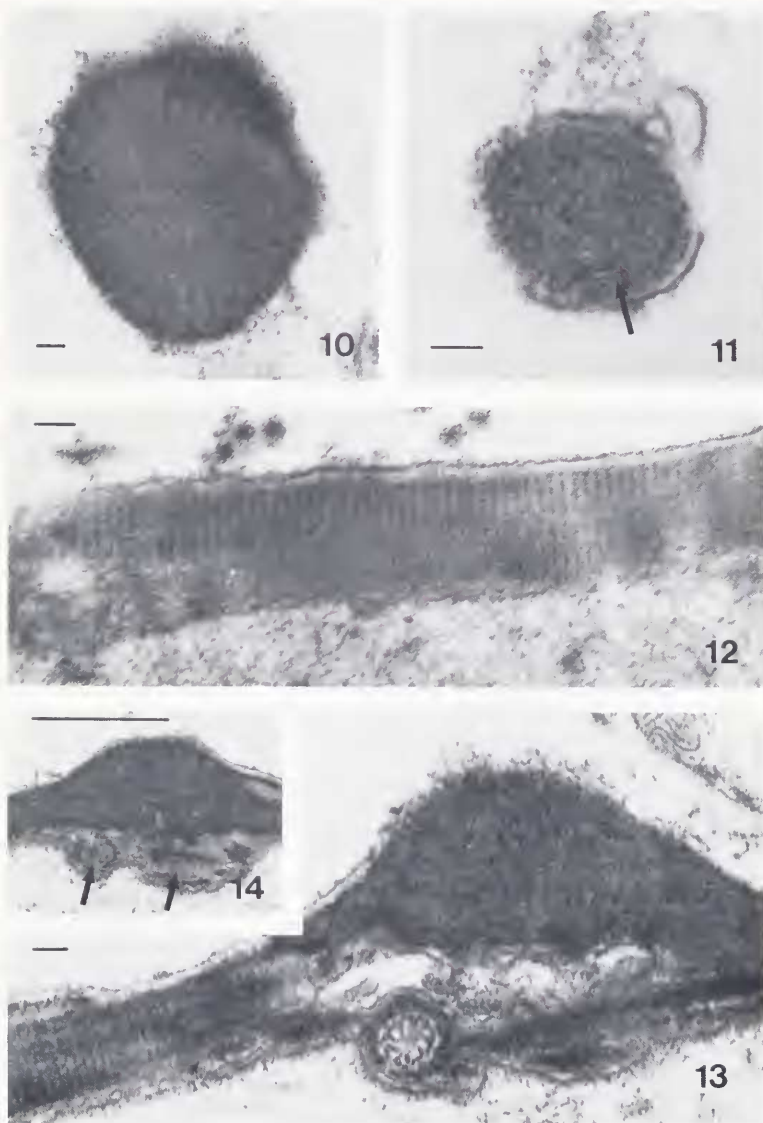
FIGURE 10. A cross section through the base of the sperm spike demonstrates the slightly compressed cross striation (due to the angle of the section) of the anastomosing radial fibrils. Bar = 0.1 μm.

FIGURE 11. At the tip of the spike cross sections reveal microtubular structures (arrow) in cross section. Similar structures have not been observed in longitudinal sections. Bar = 0.1 μm.

FIGURE 12. The radial fibrils of the cap-like region of the base show the same cross-striated appearance and presence of 6 nm filaments as are present in the sperm spike. Bar = 0.1 μm.

FIGURE 13. Interconnecting fibrils between the radial fibrils of the base and the centriolar matrix are also observed in cross section of the sperm base and contain cross striations and filaments as observed in the spike and the radial fibrils. Bar = 0.1 μm.

FIGURE 14. Centrioles are located in the main body of the sperm (arrows) and as shown in this section are housed acentrically at the base of the spike. Bar = 1.0 μm.

aldehyde. These whorls appear to be equivalent to the perinuclear vesicular region described in the sperm of *Palaemonetes paludosus* (Brown, 1967; Koehler, 1979). Koehler (1979) identified a PAS positive reaction in this region of the sperm and
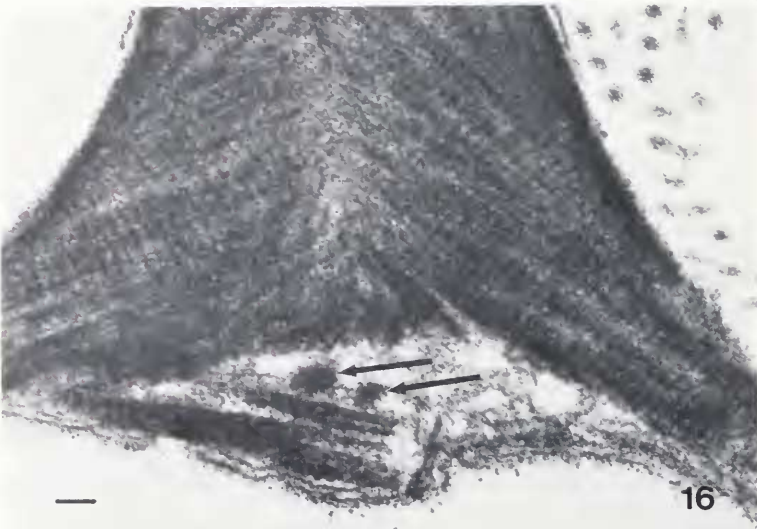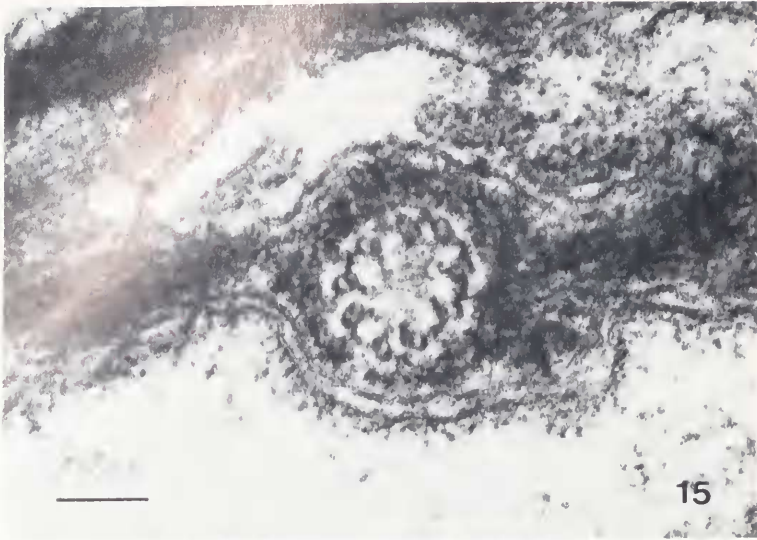
FIGURE 15. Nine doublet tubules comprise the centrioles as shown at high magnification in transmission electron micrographs. Notice the interconnecting fibrils intersecting the centriolar matrix. Bar = 0.1 μm.

FIGURE 16. A centriole in longitudinal section is often associated with dense bodies (arrows). These dense bodies probably represent cross sections through the interconnecting fibrils. Bar = 0.1 μm.

thus suggested it was acrosomal in nature. This suggestion is questionable, however, since fertilization studies have demonstrated the spike is the leading edge of the sperm during investment coat penetration (Lynn and Clark, 1983).

In the natantians, the spike has been suggested to be part of the acrosome (Pochon-Masson, 1969; Lu, 1976; Kleve *et al.*, 1980), a role also suggested for the cross striated structures associated with the isopod and schizopod sperm (Reger and Fain-Maurel, 1973) and the cross striated structure which forms a part of the ac-

rosomal complex in the octopus (Galangau and Tuzet, 1968). Pochon-Masson (1969) refers to the *Palaemon elegans* sperm spike as the acrosome and percutor organ based on analogies to the reptantian sperm. No acrosome reaction or characteristic histochemical staining, however, was observed in *P. elegans*. The spike of *Sicyonia ingentis* and *Penaeus aztecus* sperm has been demonstrated to be a part of an elaborate acrosomal complex (Lu, 1976; Clark *et al.*, 1980; Kleve *et al.*, 1980; Clark *et al.*, 1981) based on observations both *in vitro* and during the fertilization process (Yudin *et al.*, 1979; Clark *et al.*, 1980; Kleve *et al.*, 1980; Clark *et al.*, 1981) and may represent the normal function of the spike in the penaeid shrimp sperm. The structure of the sperm spike in *M. rosenbergii* is dissimilar to the structure observed in *P. aztecus* and *S. ingentis*, however, and its possible role as an acrosome is still uncertain since an acrosomal reaction has not been observed in the Macrobrachium cell (Lynn, 1981), although a dramatic spike bending process is observed during fertilization (Lynn and Clark, 1983).

The cross striated appearance of the spike and the radial fibrils, the numerous 6 nm filaments which run the length of these structures, and the unique spike bending process involved in fertilization clearly define the spike complex as the most unusual feature of the *M. rosenbergii* sperm. In agreement with other authors (Lu, 1976; Talbot and Summers, 1978), the present study suggests that the spike of the caridean sperm is not analogous to the spikes of the reptantian sperm, since *M. rosenbergii* sperm appendages contain neither nuclear material nor extensive microtubular complexes. Although microtubules may be present in cross sections of the tip of macrobrachium sperm spikes, they are not observed in longitudinal sections and certainly do not represent as prominent a feature as in some reptantian species.

Cross-striations similar to those observed in the spikes of Macrobrachium sperm have also been reported in the sperm of *P. elegans* (Pochon-Masson, 1969) and *P. paludosus* (Koehler, 1979; Brown, 1967). In contrast to the 35 nm periodicity in *M. rosenbergii* sperm, both Koehler and Pochon-Masson report the periodicities of the striations as 22–26 nm. All three species, however, do contain 4–6 nm filaments running parallel to the length of the spike. Similar filaments are also observed in the radial fibrils and resemble microfilaments. Currently the function and biochemical composition of the spike other than its proteinaceous nature is uncertain.

It is interesting that centrioles are not observed at the base of the spike in the *P. aztecus* and *S. ingentis* sperm, whereas a pair of centrioles are closely associated with the base of the spike and radial fibrils in Macrobrachium sperm. In contrast to a typical centriole, however, centrioles in Macrobrachium sperm consist of nine doublets with dynein-like arms and no central axis structures. By comparison, a typical centriole is composed of nine triplets, occasionally with internal axis spokes (McNitt, 1974). Koehler (1979) reports no centriolar structures in the mature sperm of *P. paludosus*, although centrioles are observed in *P. elegans* and *Crangon vulgaris* (Pochon-Masson, 1968a, 1969). Similar unusual centrioles have been demonstrated in the sperm of *Eupagurus bernhardus* (Pochon-Masson, 1963), several species of isopod sperm (Cotelli and Lanzavecchia, 1972; Cotelli *et al.*, 1975), and in somatic cells (Hoage and Kessel, 1968). Although no specific function has been attributed to these centrioles, they may act as control centers for contractile processes (Salisbury and Floyd, 1978; Kleve and Clark, 1980).

Two structures which have been observed in association with the centrioles of *M. rosenbergii* are intersecting fibrils and "dense bodies" resembling satellites. The actual identity of the latter is uncertain since no microtubules are associated with them and they are located closer to the centriole than is normal for satellites (Kleve

and Clark, 1980). It would appear that these "dense bodies" are actually sections of fibrils intersecting the centriolar matrix. These fibrils contain the same striated pattern and periodicity as is seen in the spike and radial fibrils. The association with the centrioles and their morphology are suggestive of ciliary rootlets. Although Fawcett and Porter (1954) report considerable variation in the periodicity of ciliary rootlets, it is usually greater than that seen in the Macrobrachium sperm structure.

Three functions have been suggested for centrioles and their associated structures in flagellate sperm; an organization center for the production of the sperm flagellum; as a division center after incorporation of the sperm into the egg at fertilization (Monroy, 1965); and, as a contractile center for altering sperm symmetry (Kleve and Clark, 1980). The presence of centrioles in a non-flagellated sperm poses a question as to their function. Obviously, they do not act as a polymerization center for flagella since none are present. It might be speculated that they are utilized as a division center in the egg after fertilization as has been suggested for a number of flagellated sperm (Monroy, 1965). Centrioles are absent, however, in several crustacean sperm (Yasuzumi, 1960; Chevaillier, 1966; Koehler, 1979). Finally, the arrangement of the centrioles and accessory fibrils from the radial fibrils might be indicative of contractile ability. The morphology of the spike and fibrils are reminiscent of ciliary rootlets, and numerous 6 nm filaments resembling microfilaments are present within the spike and fibrils. Although other sperm are reported to contain actin microfilaments (Tilney *et al.,* 1973; Brown, *et al.,* 1976) current electrophoretic and immunological evidence suggests actin is not present in *M. rosenbergii* sperm (Lynn, 1981). The question of contractile ability is still intriguing, however, for two reasons. First, the fibrils do resemble centriolar rootlets. Second, while the above functions were attributed to actin, there is increasing evidence suggesting non-actin proteins may also be involved in cell movement (Klass and Hirsch, 1981).

Obviously, the sperm of *M. rosenbergii* display a highly unusual organization. Several questions concerning the function of the sperm structures have been raised. These questions relate to the presence of an acrosome and the role of the spike and radial fibrils in their association with the centrioles. Observations on fertilization are essential in further looking at these questions and are reported in a following paper.

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